Selective delivery of dexamethasone to inflamed endothelium via E-selectin

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Summary, discussion and perspectives

Summary

This thesis describes the selective delivery of dexamethasone to activated endothelial cells, using E-selectin as a target molecule. Activated endothelial cells play an important role in the pathophysiology of chronic inflammatory diseases and are therefore considered valuable targets for pharmacological intervention. Dexamethasone is a corticosteroidal anti-inflammatory drug with a wide spectrum of pharmacological effects and is therapeutically used in a variety of chronic inflammatory diseases. However, its use is associated with serious side effects such as osteoporosis and diabetes, thus hampering long-term systemic application. Cell selective delivery of dexamethasone to activated endothelial cells might circumvent these problems.

Little is known about targeting drugs to activated endothelial cells in general and corticosteroids in particular. This thesis describes the development and characterization of drug targeting preparations that contain dexamethasone as a model anti-inflammatory drug and anti-E-selectin antibodies as a homing device. An immunoconjugate as well as an immunoliposomal formulation were produced and investigated. Studies included analyses of *in vitro* binding and internalization of developed preparations into activated endothelium, as well as *in vivo* accumulation in inflamed tissue. Several murine models of inflammation have furthermore been investigated regarding E-selectin mRNA and protein expression. These models may be used to examine therapeutic effects of developed drug targeting preparations.

In **chapter 1** the aim of the thesis is presented. **Chapter 2** reviews targeting of drugs to vascular endothelium in inflamed tissue. The pathophysiology of inflammation is described and possible target epitopes and targeting devices are summarized. Drugs possibly interfering with endothelial cell activation are discussed, as well as available *in vitro* and *in*

vivo techniques to study endothelial cell activation and inflammation. Finally, general considerations and practical directions are presented for targeting activated endothelial cells as a new approach for the treatment of chronic inflammatory disorders.

Chapter 3 and 4 describe the development and characterization of dexamethasone-anti-E-selectin (dexa-Ab_{Esel}) immunoconjugates. In **chapter 3**, binding and internalization of dexa-Ab_{Esel} into activated endothelial cells was qualitatively studied using a variety of techniques. These included immunohistochemistry, confocal laser scanning microscopy and immuno-transmission electron microscopy, showing that the conjugate selectively bound to and was internalized into activated endothelial cells. Furthermore, the conjugate was subsequently intracellularly routed to multi-vesicular bodies, which are lysosomal compartments of the cell. Degradation of intracellularly delivered dexa-Ab_{Esel} resulted in release of pharmacologically active dexamethasone, as shown using a GRE (glucocorticoid responsive element) driven reporter assay and Northern blot analysis of IL-8 mRNA levels.

In **chapter 4**, radioactively labeled dexa-Ab_{Esel} was used to quantitatively determine the kinetics of binding and internalization of dexa-Ab_{Esel} into activated endothelial cells. This was compared to the cellular uptake of unconjugated dexamethasone, which can readily pass cellular membranes without the need for a (saturable) carrier system such as E-selectin. It was shown that, upon incubation with concentrations unconjugated dexamethasone exceeding 3 μM, higher intracellular levels of the drug were achieved compared to dexa-Ab_{Esel}. However, the conjugate has the advantage of selectively delivering dexamethasone into activated endothelial cells and not into resting endothelial cells. This characteristic will avoid dexamethasone-associated side effects in other cell types of the body. Furthermore, the amount that can be intracellularly delivered using dexa-Ab_{Esel} is sufficient for pharmacological effects of dexamethasone *in vitro*, as was demonstrated in chapter 3 by inhibition of IL-8 mRNA expression by activated endothelial cells.

In **chapter 5**, dexa-Ab_{Esel} immunoconjugates were compared with Ab_{Esel}-immuno-liposomes containing dexamethasone-phosphate with regard to binding and internalization *in vitro*. It was shown that the immunoconjugates were internalized by activated endothelial cells to a larger extent than the immunoliposomes. However, due to their high drug loading capacity, immunoliposomes will in theory be able to deliver more dexamethasone in the cells.

The body distribution of both drug targeting preparations was studied in a murine delayed type hypersensitivity model, in which E-selectin is expressed by activated endothelial cells in inflamed skin. Both dexa-Ab_{Esel} and Ab_{Esel}-immunolipomes were detected in endothelial cells in inflamed DTH skin, although Ab_{mEsel}-liposomes could also be clearly visualized in the unaffected skin. The reason for the latter observation remains obscure. In addition, dexa-Ab_{Esel} could not be detected in organs like brain, lung, kidney and liver, whereas liposomes were detected in non-parenchymal cells of the liver and the spleen, representing

cells of the reticulo-endothelial system. This accumulation in non-target cells may lead to the emergence of side-effects of the immunoliposomal drug-targeting preparation, although the balance between effects and side-effects may still be favorable compared to untargeted dexamethasone. Future studies investigating anti-inflammatory effects in appropriate models of inflammation should reveal the therapeutic possibilities of both types of drug targeting systems for the treatment of chronic inflammatory disorders.

In chapter 5 the delayed type hypersensitivity model was used to determine homing capacities of developed drug targeting preparations. However, the delayed type hypersensitivity model is a transient model, which means that the disease resolves by itself in a short period of time. Using E-selectin directed drug targeting strategies, the targeted drug will be delivered at a stage in the inflammatory process when interference with endothelial cell activation may be too late for therapeutic effects. Hence, it is anticipated that a chronic model of inflammation is necessary to study therapeutic effects of the developed drug targeting preparations. Therefore, in **chapter 6**, several murine models of inflammation were investigated regarding E-selectin expression, both on mRNA and protein level. Whereas mRNA levels are indicative of gene transcriptional activity, transmembrane expression of E-selectin by the activated endothelial cells is a prerequisite for specific homing of E-selectin directed drug targeting preparations.

We showed that in all models E-selectin mRNA could be detected. In contrast, using three different antibodies that recognize different epitopes of the E-selectin molecule, E-selectin protein expression could not always be visualized. This stresses the need to determine E-selectin expression at the protein level and not at the mRNA level only, before choosing a model for studying therapeutic effects of E-selectin directed drug targeting preparations. These observations also emphasize the importance of the choice of the antibody to be used as a homing device in relation to the animal model under study. In addition, when using C57Bl/6 mice, the particular tissue affected was a determinant in whether E-selectin protein could be detected or not (skin versus aorta). This suggests spatiotemporal and/or pathophysiological determined differences in expression of different splice-variants of E-selectin by micro- and macrovasculature, as has been described for rats and humans.

Together, the results presented in this thesis demonstrate the potential of targeting dexamethasone into activated endothelial cells via E-selectin. Using an immunoconjugate, dexamethasone can be intracellularly delivered *in vitro*. Immunoliposomes, which have a high dexamethasone loading capacity, are also selectively internalized by activated endothelial cells. Both these drug-targeting constructs accumulated in activated endothelial cells in inflamed skin *in vivo*, although the immunoconjugate showed more selective targeting than the immunoliposomes. Future studies using murine models of chronic inflammation will have to elucidate the therapeutic perspectives of drug-targeting strategies to activated endothelial cells.

Discussion and Perspectives

In general, drug-targeting constructs consist of a drug, a homing device and a carrier molecule. The carrier molecule can also have intrinsic homing capacities, as is the case in e.g. immunoconjugates where the drug is directly coupled to an antibody. In the here-presented thesis research, dexamethasone was used as a model anti-inflammatory drug in the protein based drug-targeting constructs. In addition to its broad anti-inflammatory effects, dexamethasone was chosen as a model drug because of the availability of an anti-dexamethasone antibody and an HPLC analysis system to characterize the immunoconjugates. Both free and conjugated dexamethasone demonstrated pharmacological effects in activated endothelial cells *in vitro*, as demonstrated by the partial inhibition of IL8 mRNA expression. Effects of (conjugated) dexamethasone on activated endothelial cells were investigated in more detail using a cDNA expression array, showing inhibitory effects on expression of for instance TGFβ, IFNβ and CD40L, in addition to IL-8 inhibition (S.A. Ásgeirsdóttir et al, submitted). However, it is not fully understood whether these pharmacological effects also occur *in vivo* and, if so, to what extent they contribute to the anti-inflammatory effects of dexamethasone. It has been described in literature that glucocorticoid treatment in patients with inflammatory bowel disease results in upregulation of IκB (inhibitor of NFκB) in endothelial cells [1]. Whether this is a direct consequence of dexamethasone interaction with endothelial cells or an indirect consequence of inhibition of leukocyte activation remains unclear, also because endothelial cells from different sources respond differently to glucocorticoids *in vitro* with regard to increased IκB expression [2, 3]. Only *in vivo* analysis of therapeutic effects of targeted of dexamethasone in chronic inflammatory disease will reveal whether this strategy will result in reduction of leukocyte recruitment and hence improvement of the diseases state.

Cellular adhesion molecules are considered critical mediators in the recruitment of leukocytes into inflamed tissue. Pharmacological agents that would prevent the induced expression of one or more of the cell adhesion molecules on the endothelium likely attenuate this inflammatory responses. Hence much effort has been put into the development of specific inhibitors of these molecules, such as for instance ICAM-1 antisense molecules, which showed profound anti-inflammatory effects in laboratory animals [4]. The observation that in a placebo-controlled trial in patients with active Crohn's disease anti-ICAM-1 antisense treatment results were disappointing is possibly due to redundant leukocyte recruitment mechanisms [5].

Despite the pharmacological effects on the above described genes, dexamethasone did not reduce adhesion molecule expression in activated endothelial cells *in vitro*, in contrast to several other inhibitors of signal transduction pathways (Figure 1).

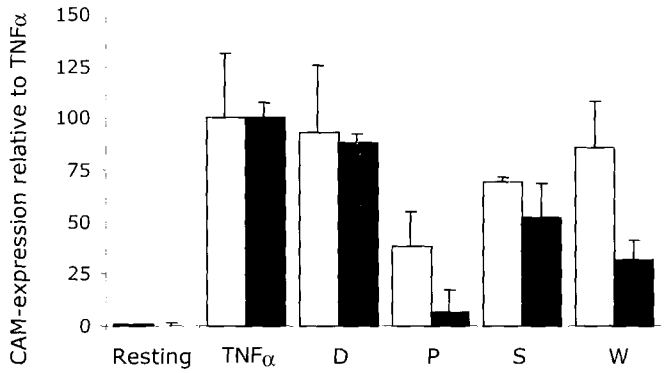

Figure 1 *Effects of inhibitors of signal transduction on HUVEC ICAM-1 (white bars) and VCAM-1 (black bars). HUVEC were treated for 24 h with 100 ng/ml TNFα together with 10 μM dexamethasone (D), parthenolide (P), SB203580 (S) or wortmannin (W). Cells were harvested, stained for ICAM-1 or VCAM-1 expression and analyzed using flow cytometry. Bars represent mean ± sd (n=3). (Figure reproduced from S.A. Ásgeirsdóttir et al, submitted)*

From these data it can be concluded that, when aiming at downregulation of CAM expression, compounds that inhibit NFκB (parthenolide), p38 MAPkinase (SB203580) or PI3-kinase (wortmannin) are suitable candidates for selective targeting to activated endothelium. Because these compounds inhibit both ICAM-1 and VCAM-1, as well as cytokines like IL-6 and IL-8 (data not shown), the problem of redundant leukocyte recruitment mechanisms will likely be reduced.

It must be kept in mind, though, that candidate drugs must meet certain requirements regarding their chemical structure and hydrophilicity. The chemical structure needs to contain functional groups that can be used for immunoconjugate synthesis, such as a hydroxyl-, amine- or carboxyl-group. From the perspective of the signal transduction inhibitors described above, only wortmannin is suitable for covalent coupling to an antibody [6]. Parthenolide and SB203580 would not be suitable because they lack proper functional groups. However, drugs derived from combinatorial chemistry procedures usually belong to a family of structurally related compounds, which have the same pharmacodynamic profile but slightly different molecular structures. This is for instance the case for the p38 MAPkinase inhibitor SB203580, which is structurally related to the compound SB202190. The latter compound can in theory be coupled to a protein as it

contains a hydroxyl group in its structure. Covalent attachment of drugs to protein carriers will allow intracellular release of the drug and hence local pharmacological effects, as demonstrated by the inhibition of IL-8 mRNA expression by conjugated dexamethasone.

The hydrophilicity of these compounds may be important in the development of liposomal drug formulations. In theory, both hydrophilic and hydrophobic compounds can be included in liposomes, by incorporation in the aqeous inner space or in the liposomal membrane, respectively. However, spontaneous release of the compound from the liposomes remains a possibility, and will always have to be determined with regard to each liposome preparation. Also in this case, the existence of structurally related compounds with slightly different physicochemical properties may be helpful in the development of stable, liposome based, drug targeting preparations. In this thesis the pharmacological effects of dexamethasone-phosphate entrapped in immunoliposomes have not been investigated. It is therefore not known whether incorporated dexamethasone will be intracellularly released, after internalization of the liposomes in the activated endothelial cells. However, the toxicity of doxorubicin-loaded liposomes to activated endothelial cells *in vitro* described in literature [7], suggests proper degradation of the liposomes, and subsequent release and pharmacological effects of targeted drug. Nonetheless, the efficacy of these intracellular processes will also be dependent on the ability of the drug to pass cellular membranes, and may thus be different for different compounds.

In general, newly developed therapeutic modalities may have favorable pharmacological properties *in vitro* as well as *in vivo*, but their pharmacokinetic profiles or side-effects may impede their further development into clinically useful compounds. This is especially the case for therapeutic proteins, gene expression regulating medicines (e.g. antisense) and gene therapy. In these cases, drug targeting may prove to be a useful tool to overcome these difficulties [8].

In this research project the drug-targeting constructs contained an anti-E-selectin antibody (Ab_{Esel}) as a homing device, which also functioned as the carrier molecule in the case of the dexa-Ab_{Esel} conjugates. The used murine anti-human E-selectin antibody recognizes the binding domain for carbohydrate ligands. Hence, the antibody will compete with leukocytes for E-selectin binding, thereby reducing the number of leukocytes that can be recruited. This may contribute to the anti-inflammatory effects of the drug targeting preparations. The use of this particular antibody, in both dexa-Ab_{Esel} and Ab_{Esel}-liposomes, thus represents a dual targeting strategy in which respectively the carrier molecule and the homing device, in addition to the delivered drug, can exert a therapeutic effect. The used antibody is however not suited for future clinical use, since murine antibodies have short circulating half-lives in humans and induce human anti-mouse antibody responses. Fortunately, advanced molecular biological techniques have created the opportunitiy to produce chimeric or humanized antibodies that, when clinically applied, have a reduced immunogenicity while maintaining their E-selectin blocking function.

Instead of antibodies, peptides recognizing E-selectin can be used as a homing device to avoid human anti-mouse antibody responses. The advantage of some peptides described in literature is furthermore the recognition of human, rat and murine E-selectin molecules [9, 10]. This is in contrast to monoclonal anti-E-selectin antibodies, which lack species cross-reactivity. Initial studies in our laboratory in which E-selectin recognizing peptides were coupled to protein backbones showed binding of the resulting constructs to an immobilized IgG E-selectin fusion protein. However, using immunohistochemistry, the conjugates were shown to bind to both resting and activated endothelial cells, indicating loss of E-selectin specificity (R.J. Kok, unpublished observations). Based on this result, we decided to always analyse binding characteristics of developed drug targeting constructs to activated endothelial cells in culture instead of studying only binding to isolated E-selectin. Furthermore, constructs should be tested in animal models as soon as possible for homing capacity to the chosen target molecule. Only this parameter will indicate the potential of the developed drug targeting construct to target activated endothelial cells in inflamed tissue.

In contrast to targeting relatively large organs like liver, kidney or tumors, the targeting of drugs to activated vascular endothelium is difficult to quantify with the use of radiolabeled constructs. This is especially troublesome because endothelial cells constitute only a small percentage of the total cell population in a particular tissue. The accumulated amount of a radiolabeled drug targeting construct in activated endothelial cells will hardly exceed the levels of radiolabeled constructs in the blood in the particular organ. In addition, it is difficult to distinguish selectively bound radioactivity from accumulated radioactivity due to enhanced permeability at the site of inflammation (unpublished observations). As a solution to these problems, the use of a dual labeling technique can be recommended, where accumulation of a ^{125}I-labeled binding construct is compared to a simultaneously administered ^{131}I-labeled non-binding control construct [11]. However, extrapolation of the accordingly determined accumulated amount to therapeutic effects is not possible without knowledge of *in vivo* pharmacokinetic-pharmacodynamic relationships of developed drug targeting constructs. Until these relationships are clarified, direct determination of pharmacological effects of newly developed drug targeting preparations will therefore remain of prime importance to determine the therapeutic success of the drug targeting strategy.

It must be realized that the added therapeutic value of targeting drugs to activated endothelial cells still needs to be determined. It has not been established yet, whether chronic inflammation will be resolved by inhibiting endothelial cell activation. This question can be addressed with the development of potent drug targeting constructs and availability of proper read-out parameters. Nowadays, RT-PCR is widely used to study pharmacological effects of drugs on the expression of susceptible genes. Because endothelial cells constitute only a small number of the total cell population in a particular tissue, isolation of activated endothelial cells from the affected organ will be obligatory to

study the effects of the delivered drug at the endothelial cell level using RT-PCR. This isolation can be performed using advanced techniques such as laser dissection microscopy. Gene expression array analysis can also be applied to determine which genes in endothelial cells are up- or downregulated by the delivered drug. However, because the yield of isolated mRNA will be relatively low using laser dissection microscopy, linear amplification of mRNA will be obligatory to detect transcriptional levels of less abundant gene transcripts. Alternatively, in situ hybridisation may be considered as a useful technique to determine pharmacological effects on gene expression. Using this procedure endothelial cells can be visually identified because the morphology of the tissue under study is retained. However, this technique has less amplification power compared to RT-PCR, which may implicitly lead to less sensitivity of the determination of eventual pharmacological effects.

After determination of pharmacological effects at the endothelial cell level, clinical parameters such as joint swelling in the case of rheumatoid arthritis need to be established to determine the therapeutic impact of the strategy.

The developed E-selectin directed drug targeting constructs described in this thesis have not been investigated yet regarding their therapeutic applications; only pharmacological effects of the dexa-Ab_{Esel} conjugates have been demonstrated *in vitro*. Further research is required to determine the potential of the developed drug targeting preparations to exert anti-inflammatory effects *in vivo*, also during chronic administration. Lastly, it should be kept in mind that targeting drugs to activated endothelial cells may prove to be an important pharmacological tool to clarify the role of the endothelium in pathophysiological processes in inflammation. This knowledge will eventually contribute to the development of novel therapies for chronic inflammatory diseases.